Inosine triphosphatase polymorphisms and ribavirin pharmacokinetics as determinants of ribavirin-associate anemia in patients receiving standard anti-HCV treatment.

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TITLE: Inosine triphosphatase polymorphisms and ribavirin pharmacokinetics as determinants of ribavirin-associate anemia in patients receiving standard anti-HCV treatment.

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SHORT TITLE: ITPA SNPs and RBV PK predict anemia.

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List of Abbreviations: HCV, Hepatitis C virus; SVR, sustained virological response; PEG/IFN, pegylated interferon; RBV, ribavirin; Hb, haemoglobin; Pk, pharmacokinetic; ITPA, inosine triphosphatase; SNPs, single nucleotide polymorphisms; TDM, therapeutic drug monitoring; IQR, inter-quartile range; ROC, receiver operating characteristic.

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Abstract.

Background. Functional variants of inosine triphosphatase (ITPA) were recently found to protect against ribavirin (RBV)-induced hemolytic anemia. However, no definitive data are yet available on the role of plasma RBV concentrations on Hb decrement. Moreover, no data have been published on the possible interplay between these two factors.

Methods. A retrospective analysis included 167 patients. The *ITPA* variants rs7270101 and rs1127354 were genotyped and tested using the χ^2-test for association with hemoglobin (Hb) reduction at week 4. We also investigated, using multivariate logistic regression, the impact of RBV plasma exposure on Hb concentrations.

Results. Both SNPs were associated with Hb decrease. The carrier of at least one variant allele in the functional ITPA SNPs was associated with a lower decrement of Hb (-1.1 g/dL), as compared to patients without a variant allele (-2.75 g/dL; $p=4.09\times10^{-8}$). RBV concentrations were not influenced by ITPA genotypes. A cut-off of 2.3 μg/mL of RBV was found to be associated with anemia (Area-Under-ROC=0.630, sensitivity=50.0% and specificity=69.5%, p=0.008). In multivariate logistic regression analyses the carrier of a variant allele (p=0.005) and plasma RBV concentrations below 2.3 μg/mL (p=0.016) were independently associated with protection against clinically significant anemia at week 4.

Conclusions. Although no direct relationship was found between ITPA polymorphisms and plasma RBV concentrations, both factors were shown to be significantly associated with anemia. A multivariate regression model based on ITPA genetic polymorphisms and RBV trough concentration was developed for predicting the risk of anemia. By relying upon these two variables, an individualized management of anemia seems to be feasible in recipients of PEG/IFN-RBV therapy.

INTRODUCTION.

Hepatitis C virus (HCV) is one of the major causes of liver cirrhosis and hepatocellular carcinoma [1]. Sustained viral response (SVR), defined as undetectable plasma HCV RNA 24 weeks after the end of treatment, is achieved in less than 50% of patients with genotype-1 infection using the currently recommended regimen consisting of pegylated-interferon (PEG/IFN) and ribavirin [2].

Anemia is a major untoward effect of anti-HCV chronic therapy. Both PEG/IFN and ribavirin (RBV) play a role in haemoglobin (Hb) decrease, although most of the responsibility is by far attributable to the latter. Moreover, with the forthcoming introduction of the new HCV protease inhibitors, telaprevir and boceprevir, the incidence of anemia is likely to increase, as shown by the results of recent clinical trials [3-5].

Repeatedly, individual RBV dose and pharmacokinetic (Pk) exposure have been found to be consistent determinants of treatment-associated anemia, and recommendations on dose reduction have been released in order to mitigate thw impact of RBV on the severity of anemia [6-10]. Some other studies have not observed an association between hemoglobin decline and plasma RBV concentrations, when multivariate analyses were performed [11-13].

Among the non-modifiable individual factors also contributing to anemia development in recipients of anti-HCV therapy, single nucleotide polymorphisms (SNPs) in the human DNA region coding for inosine triphosphatase (ITPA) were identified recently as the most significant variables influencing the risk of anemia. In these studies, by using a genome-wide association approach, two functional variants of ITPA, including one coding and one intronic variant, were found to be associated with treatment-induced anemia in HCV-infected patients [5, 14, 15], as defined by the magnitude of Hb reduction after 4 weeks of treatment. This genetically determined vulnerability was subsequently confirmed in several ethnically heterogeneous clinical cohorts. The association signal was accounted for by 2 functional variants in the *ITPA* gene on chromosome 20: a missense variant in exon 2 (rs1127354, P32T) and a splice-altering single nucleotide polymorphism in intron 2 (rs7270101). Both polymorphisms had previously been well characterized and validated as functional variants in studies of patients with ITPase deficiency, a benign inherited enzymopathy in which inosine triphosphate (the substrate for ITPase) accumulates in red blood cells [16, 17].

A recent study established that intraerythrocytic accumulation of ITP, as seen in carriers of such anemia-protecting mutants, provides an alternative source of nucleoside-triphosphates that eventually compensates for RBV-induced ATP reduction [18].

Since it is not known whether any direct relationship exist between ITPA polymorphisms and RBV pharmacokinetics, the effects of ITPA genetic variants (rs7270101 and rs1127354) and RBV trough concentration on the development of ribavirin-associated anemia at week 4 were retrospectively evaluated in HCV-mono-infected patients who underwent PEG-IFN/RBV therapy, and had complete medical records as well as samples available for genetic and pharmacokinetic analyses. As a simultaneous evaluation of ITPA SNPs and plasma RBV concentrations for hemolytic anemia has not yet been published, we aimed to understand the contribution of each factor and any possible interplay between them.

PATIENTS AND METHODS.

Patients

In this retrospective study, 234 patients with chronic HCV infection, treated in two university hospitals (Amedeo di Savoia and S. Giovanni Battista) of the city of Turin, Italy, between March 2005 and November 2008 were enrolled.

Patients were treated with PEG/IFN-α-2b (1.5 µg/kg s.c. once a week; sub dermal injection) or PEG/IFN-α-2a (180 µg once a week; sub dermal injection) plus RBV (600–1400 mg daily depending on bodyweight; orally). Sampling was performed after obtaining written informed consent in accordance with local ethics committee guidelines. Since patients' authorization for routine sampling at the time of treatment did not include genetic analyses for polymorphisms (while therapeutic drug monitoring (TDM) of RBV is performed routinely and authorized on a regular basis since early 2005), patients were asked for an additional authorization for the genetic screening of their stored samples. Main inclusion criteria were: no concomitant interacting drugs, self-reported adherence > 95%, no RBV and/or PEG/IFN dose modification up to week 4 of treatment and no treatment with growth factor before week 4. Patients with other forms of liver disease, active hepatitis A, hepatitis B infection, HIV infection, decompensated liver disease, hepatocellular carcinoma, severe depression or other psychiatric diseases, significant cardiac or renal disease, seizure disorders or pregnancy were excluded from this study. Data collected included: age, gender, weight, previous IFN therapy, concomitant drugs, baseline and week 2 and 4 biochemical parameters, such as white blood cells, Hb, alanine transaminase (ALT) level, serum HCV RNA level (logIU/mL).

Genotyping

Patients from whom DNA samples were available and who agreed to undergo genetic analyses were genotyped at polymorphic sites rs1127354, rs7270101 and rs6051702 on chromosome 20 using the ABI TaqMan allelic discrimination kit by real time PCR using standard methodology. All primers, probes, and PCR conditions are available on request. The possible genotypes for each biallelic polymorphism are as follows: rs1127354: C/C, A/C, A/A (minor allele = A); rs7270101: A/A, A/C, C/C (minor allele = C); rs6051702: A/A, A/C, C/C (minor allele = C).

Measurement of plasma ribavirin concentration

At week 4, a time associated with the achievement of RBV steady-state [19], plasma samples were collected ~12 h after the dose administration of RBV, just before the following administration (trough value). Samples were centrifuged at 3000 rpm for 10 minutes to separate plasma and then stored at -80°C until analysis. Ribavirin concentrations were measured by a previously validated HPLC method [20]. Briefly, the extraction of RBV from an aliquot of 250 µL of plasma was performed using 1500 µL of acetonitrile. The mixture was vortexed for 10 seconds and then centrifuged at 12,000×g for 10 minutes at 4°C and the supernatant was dried at 50°C. The dry extract was reconstituted with 125 µL of mobile phase then 30 µL was injected onto the column. Chromatographic separation was performed by an Atlantis 3µm dC18 column (150mm×4.6mm I.D, Waters, Milan, Italy), and RBV peak was detected at a wave length of 235 nm and retention time of 4.3 minutes. The calibration curve ranged from 0.078 to 10 µg/mL. Accuracy and precision standard errors were below 10%.

Definition of Clinical End Points

In common with most therapeutic studies on HCV chronic hepatitis treatment, Hb decline at week 4 was taken as the clinical endpoint and patients were classified according to two clinical cut-off: (a) absolute Hb value lower than 10 g/dL, and (b) Hb reduction >3 g/dL at week 4.

Statistical Analysis

For descriptive statistics, continuous variables were summarized as median (25th to 75th percentiles). Categorical variables were described as frequency and percentage. All data were assessed for normality using a Shapiro-Wilk test and categorical data were compared using a Mann Whitney or Kruskal-Wallis statistical test. To investigate continuous data, a Spearman Rank correlation was utilized. The association between individual ITPA SNP and the incidence of significant Hb decline was tested by a basic allelic test and calculated using the χ^2-test. Receiver operating characteristic (ROC) curves were used to calculate cut-off values. Multivariate logistic regression analysis with stepwise forward selection was performed with *P*-values of less than 0.05 as the criteria for model inclusion. Statistical analyses were conducted by using SPSS software package ver. 18.0 (Chicago, IL, USA) and using Prism 5.0 (GraphPad Software, San Diego, USA). Linkage disequilibrium analysis was conducted considering r^2 and using Haploview 4.2.

RESULTS.

Sixty-seven out of 234 patients were excluded due to the lack of demographic and clinical information, including RBV plasma evaluation at week 4.

Of the 167 patients included in the analysis, 90 patients (53.9%) were male, the median age was 46 years (IQR, 38–58 years) and the median body weight was 69 kg (IQR, 62–75 kg). 161 patients (96.4%) were Caucasian and 6 (3.6%) were African. 81 patients (48.5%) were treated with PEG/IFN-a-2a (180 µg/ once a week) and 86 (51.5%) were treated with PEG/IFN-a-2b (1.5 µg/kg s.c. once a week). The median dose of RBV was 14.01 mg/kg (12.68-15.38). The majority of patients were treated with 800 mg (n=51) or 1000 mg (n=103) of RBV; three patients received 600mg, nine 1200 mg and one 1400 mg.

The median Hb reduction was -2.4 g/dL (-3.5 to -1.3) at week 4. 59 patients (35.3%) had anemia (Hb reduction>3g/dL or Hb<10g/dL) at week 4. Baseline Hb was correlated with Hb reduction (rho=-0.429 p=7.15×10^{-9}).

The variant allele frequencies for rs1127354 C/A, rs7270101 A/C and rs6051702 A/C were 4.8%, 11.1%, and 13.7%, respectively. All SNPs were in Hardy-Weinburg equilibrium. The three SNPs in analysis did not result in Linkage Disequilibrium ($r^2<0.5$) in our population. No differences concerning demographic, racial, physical characteristics and biochemical parameters, (Hb, platelet count, alanine transaminase -ALT- level, serum HCV RNA level) were observed among genetically defined groups.

The two functional ITPA SNPs, responsible for inosine triphosphatase deficiency, (rs1127354 and rs7270101) and the co-segregate SNP (rs6051702) were associated with the magnitude of hemoglobin decrease. Patients with rs1127354CC (n=151) had a larger hemoglobin decrement (-2.5 g/dL, IQR -3.6 to -1.4), as compared to patients with CA/AA genotypes (-0.8 g/dL, IQR -1.1

to -0.4, $p=3.06x10^{-6}$). Similarly patients with genotype rs7270101AA (n=133) had a larger hemoglobin decrement (-2.5 g/dL, IQR -3.6 to -1.4), as compared to patients with AC/CC (-1.7 g/dL, IQR -2.5 to -0.7, p = 0.002) and patients with rs6051702AA (n=129) had a larger hemoglobin decrement (-2.7 g/dL, IQR -3.7 to -1.7) as compared to patients with AC/CC (-1.1 g/dL, IQR -1.9 to -0.7, $p=3.4x10^{-9}$).

Despite the strong association between the hemoglobin decrement and the polymorphism rs6051702, in the following statistical analysis we have considered only the two functional polymorphisms of the gene ITPA, as described by other authors [5, 21, 22].

As showed in Figure 1, the carrier of at least one variant allele in the functional ITPA SNPs was associated with a smaller decrement of Hb ($p=4.09x10^{-8}$). In particular, patients with at least a variant allele in the functional ITPA SNPs (n=49) had a lower hemoglobin decrement (-1.1 g/dL, IQR -2.3 to -0.6), as compared to patients (n=118) without a variant allele (-2.75 g/dL, IQR -3.7 to -1.7). No differences in Hb baseline between the wild type genotype patients and the carriers of a least one mutation for ITPA gene were observed (p=0.843).

Median trough plasma RBV concentrations at week 4 were 2.1 μg/mL (1.63 to 2.68). Weight at baseline (rho=-0.286 $p=4.0x10^{-4}$) and dose/kg of RBV (rho=0.265 p=0.001) were correlated with plasma RBV concentrations.

RBV concentrations were not influenced by ITPA genotypes. Patients with rs1127354CC had a median RBV concentration of 2.1 μg/mL (1.63 to 2.68) compared with 1.76 μg/mL (1.50 to 2.6, p=0.269) in patients with the CA/AA genotype. Similarly patients with rs7270101AA had a median RBV concentration of 2.09 μg/mL (1.63 to 2.68) compared with 2.1 μg/mL (1.63 to 2.90, p=0.795) in patients with the rs7270101 AC/CC genotype. A trend towards higher RBV concentrations could be observed in patients with rs6051702AA with a median of 2.14 μg/mL

(1.68 to 2.82), while patients characterized by rs6051702 AC/CC genotypes had median of 1.83 μg/mL (1.47 to 2.20, p=0.067). Plasma RBV concentrations at week 4 were correlated with Hb reduction at week 4 (rho=-0.183 p=0.025).

To define the clinical relevance of genetic variants, trough concentration of RBV and other variables, we analyzed the proportion of patients with clinically significant anemia, which was defined as a decline of Hb more than 3 g/dL or an absolute Hb value lower than 10 g/dL. As summarized in Table 1, differences could be detected for baseline Hb, RBV trough concentrations and possession of variant alleles in the two SNPs.

By using ROC curve analysis, a cut-off of plasma RBV concentration of 2.3 μg/mL was found to be associated with anemia (Area-Under-ROC=0.630, sensitivity=50.0% and specificity=69.5%, p=0.008). Using this cut-off, 30 out of 59 (50.8%) patients with a RBV concentration above 2.3 μg/mL developed anemia, while this occurred in only 27/108 (25.0%) patients with RBV concentrations below 2.3 μg/mL ($\chi 2$=8.08, p=0.003).

Many factors previously linked to anemia were also included in multivariate logistic regression analysis. As summarized in Table 2, carrier of variant allele (OR=0.29, p=0.005) and RBV plasma concentrations below 2.3 μg/mL (OR=0.42, p=0.016) were independently associated with protection against clinically significant anemia. The probability of developing anemia based on these two predictive factors is shown in Figure 2. Considering patients with RBV concentrations below 2.3 μg/mL, the predicted probability of developing anemia was 35.5% for patients with wild type genotype for all SNPs (n=72) and 13.8% for patients with at least one SNP with a variant allele (n=31). Considering patients with RBV concentrations above 2.3 μg/mL, the predicted probability for patients with wild type genotype for all SNPs (n=47) was 56.8%, compared with 27.5% for patients with at least one SNP with variant allele (n=17).

As shown in Figure 3 carriers of ITPA variants had a smaller decrease of Hb as compared to patients with wild type ITPA genotype. In the same figure and analysis it is also apparent that, regardless of the RBV concentration, in carriers of ITPA variants the magnitude of Hb decrement was found to have no significant association with plasma RBV concentration (rho=-0.17, p=0.28), while the magnitude of Hb decrease among patients with the WT ITPA genotype had a strong and significant association with RBV concentrations (rho=-0.20, p=0.035).

DISCUSSION.

Recently several genetic variants associated with substantial effects on both efficacy and toxicity of PEG/IFN and ribavirin have been identified. SNPs in the *IL28B* gene were found to be strongly associated with response to therapy of chronic genotype 1 HCV infection [23-27], and SNPs in the *ITPA* gene were identified as predictors of RBV treatment-associated anemia in the European-American and Japanese populations [5, 14, 15, 28]. The anemia experienced as a consequence of PEG/IFN and RBV combination therapy is primarily caused by a RBV-induced haemolysis and secondarily by interferon-induced bone marrow toxicity. Ribavirin toxicity can be explained by the accumulation of RBV phosphate metabolites in erythrocytes, oxidative damage and consequent cell lysis [29]. The impact of anemia on the outcome of anti-HCV infection therapy is substantial, since RBV dose-reduction or early treatment interruption due to anemia often leads to suboptimal drug intake and a higher chance of treatment failure [30]. The possibility of identifying those patients who are more likely to undergo significant Hb loss while on treatment might be helpful in order to implement those measures which might limit the impact of anemia on treatment outcome.

In this study the newly identified genetic polymorphisms of ITPA as well as RBV pharmacokinetic exposure, using RBV trough plasma concentration, confirmed their value as predictors of treatment-induced anemia, as established by HB reduction after 4 weeks of PEG/IFN-RBV treatment. Furthermore, we observed that baseline Hb was correlated with Hb reduction. However having a higher baseline HB concentration is a biologically benign condition, which testifies to a higher functional reserve as compared to the other patients. For pure numerical reasons such condition is more vulnerable to a HB loss, since HB loss is here measured in terms of absolute values rather than percentage HB loss from baseline.

Although, in addition to ITPA polymorphisms and RBV concentration, a series of individual features such as age, platelet count, haemoglobin concentration and haptoglobin phenotype were found to have some degree of association with the risk of anemia [8, 31, 32], the only modifiable factor of these variables is RBV pharmacokinetic exposure, which shows remarkable variability among HCV-infected patients [33]. Thus, the early assessment of RBV concentration is suggested by some studies [34, 35] in order to adjust individual RBV dose.

Following the identification of ITPA polymorphism as the strongest predictor of anemia in PEG/IFN-RBV recipients, the question arises as to how to integrate this newly identified genetic marker with RBV trough concentration in order to predict and manage the individual risk of anemia. According to our results, there is no apparent correlation between ITPA polymorphisms and RBV pharmacokinetic exposure, as also expected by the reported lack of association between ITPA polymorphism and treatment outcome.

Thus, the two variables analyzed here, ITPA functional polymorphism and RBV concentration, retain their independent predictive values and display limited overlap in terms of predictive value. As shown in figure 2, the reciprocal increase in predictive value provided by either variable is significant. When relying upon RBV exposure, it is apparent that with high RBV concentration the possession of ITPA variants is associated with a lower risk of anemia as compared to patients with WT genotype and, changing the point of view, patients with the same ITPA genetic profile have a higher risk of anemia in case of higher RBV concentration.

Thus, the introduction of the described ITPA genetic polymorphism substantially improves our ability to predict the individual risk of treatment-induced anemia (perhaps also considering the non-functional polymorphism rs6051702?). The main implication of our results is that the possession of ITPA variants is associated with a rather limited median Hb loss, independent of

RBV concentration, even when RBV concentration is above the threshold of 2.3 µg/mL. In our patients anemia occurred in 8 of 49 ITPA variant carriers (16.3%) and in 51 of 118 of ITPA WT patients (43.2%). It is worth noting that the only 6 patients who had Hb value less than 10 g/dL at week 4 were all ITPA WT carriers.

In practical terms, given that human genetic analysis is easier and quicker to perform, with turnaround time shorter than TDM of RBV, and that the technique is far more widespread than TDM, the baseline estimation of the individual risk of treatment-induced anemia seems now a step closer to becoming common practice. In this perspective, RBV TDM might maintain a role in the management of the prevailing proportion of patients with WT ITPA genotype, in whom RBV concentration may be fruitfully modified by RBV dose adjustment. Since RBV pharmacokinetic exposure is also associated with the rate of SVR [6, 11, 13, 27, 36, 37], the same might also apply to carriers of ITPA variants with suboptimal RBV concentration, in whom RBV dose may be increased with a smaller risk of anemia.

In conclusion, this retrospective study confirms the high impact of ITPA SNPs on hemolytic anemia and seems to suggest a cut-off value for trough plasma RBVconcentration. Although more studies are needed to clarify and confirm the relationship between anemia, ITPA polymorphism and RBV pharmacokinetics, these results show that estimating the risk of anemia by relying upon both these two determinants was more accurate than the use of a single assessment of either variable, assuming plasma RBV concentrations are a key factor to predict anemia. Moreover, these data might form the basis for the development of an individual screening algorithm able to reduce the impact of anemia on the rate of response to anti-HCV treatment.

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Figure Legends.

Figure 1. The possession of at least one variant allele in the functional ITPA SNPs (n=49) was associated with a smaller decrement of Hb (g/dL) at week 4 ($p=4.09\times10^{-8}$). Median values (horizontal line), IQR (bars), patient values (black square), highest and lowest value (whiskers) are shown.

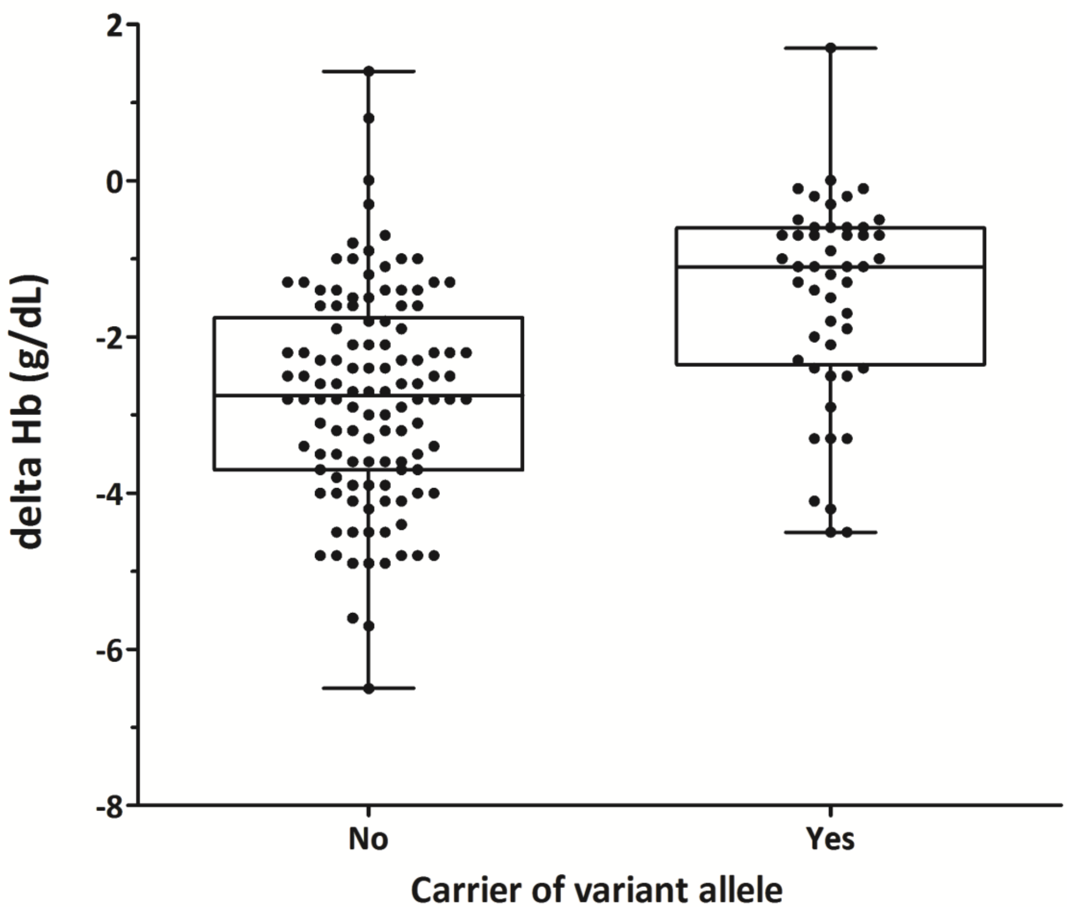

Figure 2. The predicted probability of developing anemia considering the possession of variant alleles and RBV concentrations (cut-off of 2.3 μg/mL) in multivariate logistic regression. The predicted probability of anemia is 13.8% for Yes-Carrier and Yes-RBV<2.3, 27.5% for Yes-Carrier and No-RBV<2.3, 35.5% for No-Carrier and Yes-RBV<2.3 and 56.8% for No-Carrier and No-RBV<2.3.

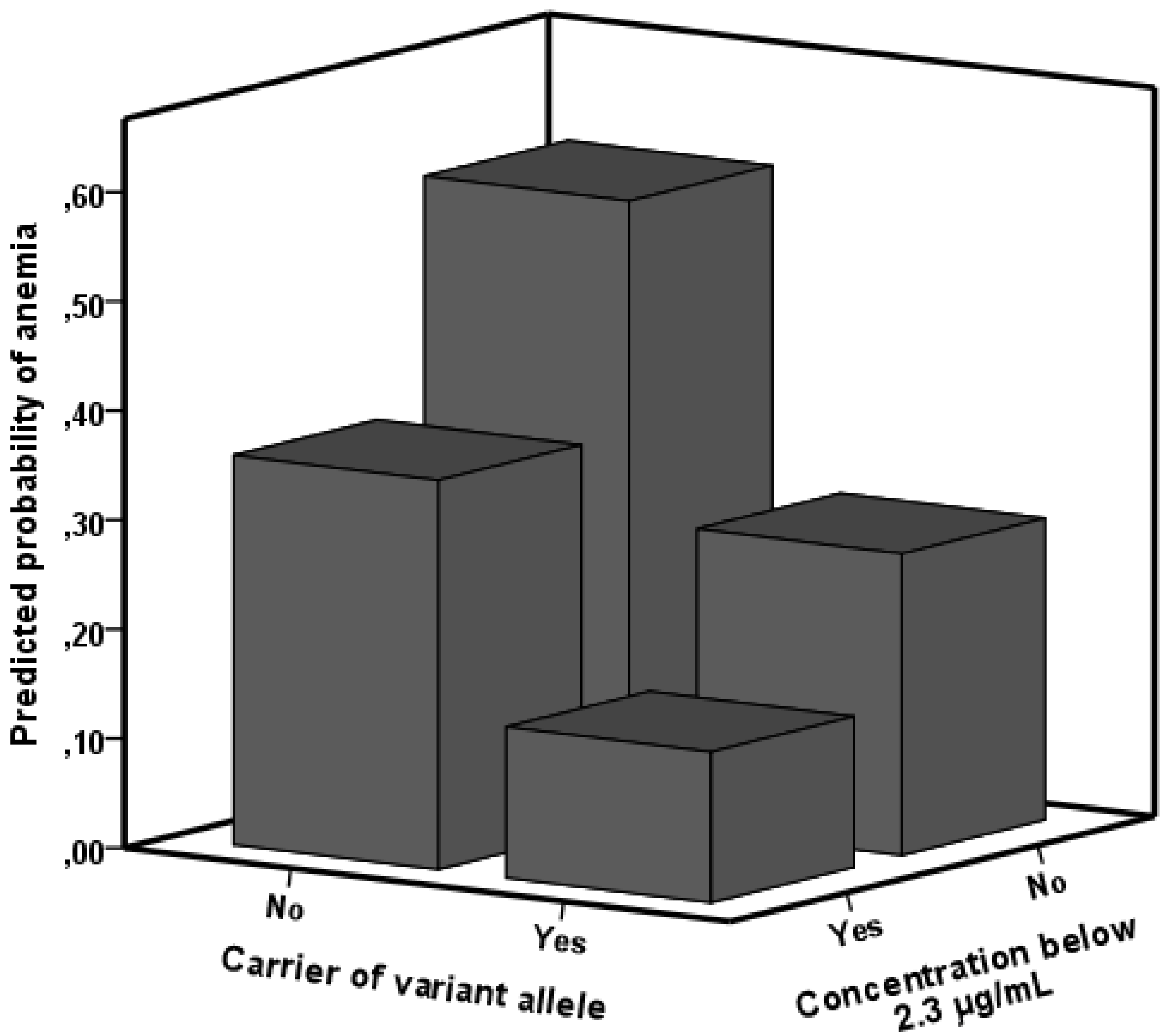

Figure 3. Scatter plot representing the correlation between RBV concentrations at week 4 (μg/mL) and Hb reduction at week 4 (g/dL). Patient values (black dots for ITPA wild type and white square for ITPA variant carrier), linear regression (filled line for ITPA wild type and dotted line for ITPA variant carrier) are shown. Patients with the wild type ITPA genotype had a strong and significant association with RBV concentrations (rho=-0.20, p=0.035).